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(54) Title: COMPOSITIONS FOR AND METHODS OF ENHANCING DELIVERY OF NUCLEIC ACIDS TO CELLS

(57) Abstract

The present invention provides compositions and methods for enhanced delivery of nucleic acids to intracellular compartments. In particular, a method of enhancing delivery and expression of nucleic acids to the nucleus is provided, and a method of enhancing delivery of nucleic acids to the mitochondria is provided, utilizing a nuclear or mitochondrial localization complex, respectively.

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COMPOSITIONS FOR AND METHODS OF ENHANCING DELIVERY OF NUCLEIC ACIDS TO CELLS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This present invention relates to delivery of exogenous nucleic acids into cells. In particular, this invention provides a means to achieve enhanced transport of transfected nucleic acids into intracellular compartments, such as the nucleus or the mitochondria.

BACKGROUND ART

The ability of DNA to charge associate with cationic substances has been documented for a number of substances including liposomes (Brigham, K.L. *et al.*, *Amer. J. Respir. Cell and Mole. Biol.* 8:209-213 (1993)) and glycoprotein complexes containing poly-L-lysine (Wilson, J.M. *et al.*, *J. Biol. Chem.* 267:11483-11489 (1992)). DNA charge interactions have also been employed to purify DNA (Richards, E.J., in *Current Protocols in Molecular Biology*, Ausubel, F.M. *et al.*, eds. pp. 2.13.1-2.13.3 (1993)).

The presence and role of protein intracellular localization signals are also documented (Adam, S. A. *et al.*, *Nature* 337(6204):276-279 (1989)). These signal peptides are used in the cell to direct newly synthesized proteins from the cytosol into the endoplasmic reticulum (ER), mitochondria, chloroplasts, and nucleus. While many of these signals are bipartite, consisting of two signal regions separated by a region of non-signal amino acid sequence, some proteins such as SV40 T-antigen have a single nuclear localization sequence (NLS) (Dingwall, C. *et al.*, *Trends in Biochemical Sciences* 16:478-481 (1991)).

The successful delivery of an exogenous gene either to eukaryotic cells in culture (*in vitro* gene transfer) or to cells in a living organism (*in vivo* gene transfer, gene therapy) can be viewed as a two-step process. First, the delivered gene must

traverse the cell membrane (go from outside to inside the cell) and second, the delivered gene must cross the nuclear or mitochondrial membrane. Several different approaches have been developed for gene transfer. The different approaches used to cross the cell membrane include the use of viral based vectors (e.g., retroviruses, adenoviruses, and adeno-associated viruses) (Drumm, M. L. *et al.*, *Cell* 62:1227-1233 (1990); Rosenfeld, M.A. *et al.*, *Cell* 68:143-155 (1992); and Muzyczka, N., *Curr. Top. Micro. Immuno.* 158:97-129 (1992)), charge associating the DNA with an asialorosomucoid/poly L-lysine complex (Wilson, J. M. *et al.* (1992)), charge associating the DNA with cationic liposomes (Brigham, K.L. *et al.* (1993)) and the use of cationic liposomes in association with a poly-L-lysine antibody complex (Trubetskoy, V.S. *et al.*, *Biochim. Biophys. Acta* 1131:311-313 (1993)).

Though the above methods attempt to improve the delivery of DNA across the cell membrane, the only approach which addresses the second step in gene delivery (crossing the nuclear membrane) has been the use of viral vectors. However, use of viral vectors has several drawbacks. These vectors use a large amount of native virus sequence to provide intracellular trafficking, with the vectors modified to be replication negative. Additionally, these methods require inserting into the viral genome the DNA to be transferred. Furthermore, both retrovirus vectors and adeno-associated viruses have low packaging efficiency with large inserts, and adenovirus vectors have been associated with significant inflammatory responses.

The present invention provides a very different, simple, highly efficient approach to transferring nucleic acids across intracellular membranes to overcome the problems in the art.

SUMMARY OF THE INVENTION

The present invention provides a purified complex comprising a nucleic acid bound to a nuclear localization peptide. The complex can further comprise a liposome and/or asialorosomucoid.

The present invention further provides a purified complex comprising a nucleic acid bound to a mitochondrial localization peptide.

The instant invention also provides a purified complex comprising a liposome, a nucleic acid and a nuclear or mitochondrial localization peptide.

The instant invention also provides a method of enhancing expression of a transfected nucleic acid in a eukaryotic cell comprising administering to the cell a purified complex comprising a nucleic acid bound to a nuclear localization peptide, thereby enhancing expression of the transfected nucleic acid in the cell.

The instant invention also provides a method of enhancing delivery of a nucleic acid to the nucleus of a cell, comprising administering to the cell a purified complex comprising a nucleic acid bound to a nuclear localization peptide, thereby enhancing the delivery of the nucleic acid to the nucleus of the cell.

The instant invention also provides a method of enhancing expression of a transfected nucleic acid in a eukaryotic cell in a subject comprising administering to the subject a purified complex comprising a nucleic acid bound to a nuclear localization peptide, thereby enhancing expression of the transfected nucleic acid in the cell.

The present invention additionally provides a method of enhancing delivery of a nucleic acid to a mitochondrion of a cell, comprising administering to the cell a complex comprising the nucleic acid bound to a mitochondrial localization peptide, thereby enhancing the delivery of the nucleic acid to the mitochondrion of the cell.

Also provided is a method of enhancing expression of a transfected nucleic acid in a cell in a plant comprising administering to the plant a complex comprising the nucleic acid bound to a plant nuclear localization peptide, thereby enhancing expression of the nucleic acid in the cell in the plant.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples included therein.

A synthetic intracellular localization peptide is used herein as part of a complex that consists of: 1) the synthetic intracellular localization peptide (also herein referred to as a "transport peptide"); and 2) a nucleic acid. The complex can also include a liposome. The general purpose of this invention is to enhance effective delivery of transfected genes into eukaryotic cells by providing a mechanism for those genes to reach and enter the cell nucleus or mitochondria. For example, increasing the percentage of transfected nucleic acids that enters the nucleus can increase expression of the transfected gene. This invention includes the use of a protein-derived, nuclear or mitochondrial localization sequence as a delivery mechanism for a nucleic acid and the use of this delivery mechanism to increase the effectiveness of gene transfer by increasing the amount of a transfected gene that reaches the cell nucleus or mitochondria.

As used in the claims, "a" or "an" can mean one or more, depending upon the context in which it is used.

Throughout this application, by "enhancing" either delivery or expression of a nucleic acid is meant that the amount of the nucleic acid delivered or the amount of expression is increased relative to the amount of delivery or expression achieved by the same method without utilizing the inventive complex that includes an intracellular localization peptide.

The present invention provides a purified complex comprising a nucleic acid bound to a nuclear localization peptide. As used herein, "nuclear localization peptide" includes any peptide having a function of enhancing delivery of a cotransfected

nucleic acid, bound to the peptide, to the nucleus of the transfected cell, as compared to the amount delivered to the nucleus when the same cell delivery method is used without the nuclear localization peptide. Several examples of such nuclear localization peptides, as well as a general description of characteristics of these nuclear localization peptides are provided herein. Additionally, these or any other potential peptides can be readily assayed by, for example, the methods described herein for assaying nuclear localization of labelled substances. Such assay is used to determine whether, when bound to a nucleic acid and administered to cells, a potential nuclear localization peptide enhances localization of the nucleic acid to the nucleus of the cells, as compared to the same type cells to which the nucleic acid without the peptide is administered by the same method. This invention provides that, typically, useful nuclear localization peptides can provide higher amounts of nucleus-localized transfected nucleic acid than other known methods of enhancing transfection, such as liposomes, asialorosomucoid or adenoviruses. Thus, useful nuclear localization peptides can typically cause higher levels of expression of transfected DNA than other known methods of enhancing transfection such as liposomes.

Throughout this application, "bound" means that the nucleic acid is complexed with the peptide, for example by charge-association between the negatively charged nucleic acid and positively charged amino acids in the peptide. A polylysine region in the peptide can be particularly useful for this charge association, for both nuclear localization peptides and mitochondrial localization peptides. Additionally, standard chemical ligation methods can be employed to link the nucleic acid to the peptide, and covalent (e.g., thioester) bonds can be formed between the nucleic acid and the protein. Such methods are standard in the art.

The inventive complex can be used to enhance effectiveness of known methods of nucleic acid delivery in cells, thus enhancing the amount of nucleic acid that is expressed by increasing the percentage of the delivered DNA that enters the nucleus. Thus, such method also enhances the delivery by known nucleic acid delivery methods of, for example, antisense RNAs, to the nucleus.

The present complex can be used in conjunction with any nucleic acid delivery method to enhance delivery of a nucleic acid to the nucleus of a cell. Therefore, the complex can comprise, in addition to the nucleic acid and the nuclear localization peptide, a liposome, such as cationic or anionic liposomes. This complex can then be used in conjunction with liposome delivery methods (e.g., Brigham, *et al.* (1993); Nicolau, C. *et al.*, *Methods in Enzymology* Vol. 149:157-176 (1987)). Additionally, when the nuclear localization peptide includes a polylysine region, the complex can further comprise asialorosomucoid, another known transfection mediator. The asialorosomucoid can bind to the nuclear localization signal via the polylysine region, and this complex can then be used in conjunction with asialorosomucoid delivery methods (Wilson, *et al.* (1992)). Additionally, the present complex can be used, for example, in methods of transfection utilizing adenoviruses (Rosenfeld, *et al.* (1992)). Furthermore, the complex can be used in conjunction with poly-L-lysine antibody complex methods which target delivery of a transgene to a cell surface receptor (Trubetskoy, *et al.* (1993)). Thus, the present complex can be utilized in cell type-targeted delivery methods, as will be elaborated upon below.

The nuclear localization peptide utilized herein can be derived from a naturally occurring nuclear localization sequence found in cells in proteins that are to be localized to the nucleus to function (e.g., Dingwall, C. and Laskey, R.A., *Annu. Rev. Cell Biol.* 2:367-390 (1986); Goldfarb, D. S., *et al.*, *Nature* 322:641-644 (1986)). However, any peptide can be tested as described herein for usefulness in enhancing delivery to the nucleus. A typical peptide can be about 4 to about 60 amino acids in length. A preferable size range is from about 4 to about 25 amino acids, and even more preferably from about 4 to about 20 amino acids. Even more preferable is about 4 to about 13 amino acids. Typically, a nuclear localization peptide contains a high concentration of positively charged amino acids residues; often it contains a high concentration specifically of lysine and/or arginine and usually it contains proline. The amino acid residues can be mutated and/or modified so long as the modifications do not affect the nuclear localization function of the peptide. Thus, as used herein, the work "peptide" includes mimetics and the word "amino acid" includes modified amino acids,

unusual amino acids, and D-form amino acids. All nuclear localization peptides encompassed by this invention have the function of localizing to the nucleus. Additionally, both straight- and branched-chain peptides can be utilized, as long as they retain this function. An example of a minimal nuclear localization peptide comprises K R/K X R/K (listed herein as SEQ ID NO:6).

In addition to the methods taught herein, as is known in the art, a nuclear localization peptide can be determined by observing its effect on the intracellular sorting of other proteins when they are attached to them by recombinant DNA methods (*see*, e.g., Lanford, R.E. *et al.*, *Cell* 37:801-813 (1984)).

Examples of nuclear localization peptides are provided in SEQ ID NOS: 1, 2, 3, 4, 6 and 7. SEQ ID NO:1 provides a typical nuclear localization peptide similar to the SV40 T-antigen nuclear localization sequence. Any nuclear localization peptide can include a polylysine tail. Therefore, SEQ ID NO:2 (termed peptide 1 herein) is the sequence of SEQ ID NO:1 with a polylysine tail. SEQ ID NO:2, for example, provides a 5-lysine polylysine tail; however, fewer or more lysines can be utilized. Typically, about 3 to about 10 lysines can be used. A preferable length is about 4-5 lysines. SEQ ID NO:3 provides yet another example of a nuclear localization peptide. It contains the amino acid residues of peptide 1, but it has a leucine residue inserted between the nuclear localization region and the polylysine tail, causing the peptide to be less linear.

Another example of a nuclear localization peptide is seen in SEQ ID NO:4, which is a sequence utilized by plants to localize proteins to the nucleus. A complex including this peptide will be particularly useful in plants.

Complete histone protein (Boehringer Mannheim Biochemicals (Indianapolis, Indiana); Calbiochem (La Jolla, California)) provides yet another example of nuclear localization peptides. In the cell, histone proteins, once they are synthesized, are localized to the nucleus. Thus, this example shows how the nuclear localization peptide from any protein that is localized by the cell to the nucleus can work in the

present method. The selected nuclear localization peptide can then be readily examined for usefulness in the present method by following the teachings herein.

As can be seen from the examples provided herein, many nuclear localization regions of proteins will be useful in the present invention. Additionally, these nuclear localization regions can be modified as desired, as long as they retain the characteristic of enhancing nucleic acid delivery to the nucleus.

The present invention also provides a purified complex comprising a nucleic acid bound to a mitochondrial localization peptide. Such a complex is useful for enhancing the delivery of the bound nucleic acid to the mitochondria of a transfected cell. Additionally, this complex can also comprise a liposome, including cationic or anionic liposomes. As for nuclear localization sequences, mitochondrial localization sequences are also known in the art, and thus, useful mitochondrial localization peptides, as determined by following the teachings herein, can be derived from, for example, proteins that are localized to the mitochondria by the cell. "Mitochondrial localization peptide," as used herein, refers to peptides having the function of directing delivery across the mitochondrial membrane into the mitochondria, as can be tested by the methods taught herein. For example, a mitochondrial localization peptide can be determined by detecting enhanced delivery of a co-transfected nucleic acid, bound to the peptide, to the mitochondria as compared to when the same delivery method is used to transfect the nucleic acid without the mitochondrial localization peptide.

Mitochondrial signals are known in the art to be characterized by being about 12-80 amino acid residues in length and to form amphipathic α-helical structures in the cytoplasm in which positively charged residues line up on one side of the helix while uncharged hydrophobic residues line up toward the opposite side. Therefore, positively charged amino acid residues are typically found periodically throughout the mitochondrial localization peptide. As described for nuclear localization signals, modified, unusual, and D-form animo acids can be utilized, and mimetics can therefore

also be utilized, as long as they retain the function of directing delivery across the mitochondrial membrane into the mitochondria.

An example of a mitochondrial localization signal is listed herein as SEQ ID NO:5. As seen in this example, a polylysine tail of a desired length, for example, from about 3 to about 10 lysine residues, can be included, for example, at the carboxy terminus of the peptide. A polylysine tail can be useful for allowing charge-association between the mitochondrial localization peptide and the nucleic acid to be delivered.

The present invention also provides a purified complex comprising a liposome, a nucleic acid and either a nuclear or a mitochondrial localization peptide. The liposome can be cationic or anionic and, if desired, the liposome can contain components to target the complex to a selected type of cell in the body, as is known in the art (Cristiano, *et al.*, *Proc. Natl. Acad. Sci. USA* 90:2122-2126 (1993); Nabel, G. J., *Proc. Natl. Acad. Sci. USA* 90:11307-11311 (1993)).

The present invention additionally provides a method of enhancing delivery of a nucleic acid to the cell nucleus and enhancing expression of a transfected nucleic acid in a cell comprising administering a purified complex comprising a nucleic acid bound to a nuclear localization peptide, thereby enhancing expression of the transfected nucleic acid in the cell.

Any cell can be transfected using techniques known in the art to transfect a selected cell type or to target a specific organ, regardless of whether a nuclear or a mitochondrial localization peptide is used. The present method can enhance the expression obtainable by the known transfection method because it increases the percentage of nucleic acid that, once it is transfected into the cell, is then localized to the nucleus. Increasing the amount of DNA that enters the nucleus can increase the number of templates available for transcription into RNA, and thus increase the number of mRNA molecules available for protein synthesis. Therefore, whether the goal of the

increased expression is to produce antisense RNA molecules or to produce a protein product, this method can enhance expression of these products.

Currently known techniques in the art for transfection of cells *in vitro* and *in vivo* include such methods as lipofection, with either cationic liposomes (such as Lipofectin, LipofectAce, etc., (BRL) (Brigham, K.L. *et al.*, *Amer. J. Respir. Cell and Mol. Biol.* 8:209-213 (1993)) or anionic liposomes (*Nicolau et al.*, (1987)). These lipofection methods can be targeted to a specific organ or cell type by methods known in the art, e.g., by site of injection or by including in the liposome structure components that direct the liposome to a specific target (e.g., *Nicolau*, C., *et al.*, U.S. Patent No. 5,017,359 (1991)).

Another useful delivery method includes the use of asialorosomucoid in a complex with poly-L-lysine and nucleic acid. This method can be used in conjunction with the present method simply by complexing asialorosomucoid with the herein described complex comprising a nucleic acid bound to a nuclear localization peptide having a polylysine tail and following the known asialorosomucoid method. Furthermore, adenovirus- or adenoassociated virus mediated transfection (Rosenfeld, N.A. *et al.* (1992); Muzyczka, N. (1992)) can also be enhanced by the present method by using in the known virus method the present complex comprising a nucleic acid bound to a nuclear or mitochondrial localization peptide.

The present method can also be used to enhance delivery/expression with known delivery methods utilizing poly-L-lysine-antibody complexes (Trubetskoy, V.S. *et al.* (1993)). This method can be achieved simply by replacing the poly-L-lysine with an intracellular localization peptide, or by adding an intracellular localization peptide to the poly-L-lysine antibody complex. Additionally, liposomes can be used in this method.

Any selected nuclear localization peptide, as defined herein, can be utilized in this method. For example, the nuclear localization peptides listed as SEQ ID NOS: 1, 2, 3, 4, 6 and 7 can be utilized. Any other selected peptide can be determined

for usefulness as described herein. The nuclear localization peptide can be chosen according to the cell type to be transfected; i.e., for mammalian cells, one can select a mammalian-derived peptide amino acid sequence and for plant cells, one can select a plant-derived peptide amino acid sequence.

The present invention also provides a method of enhancing delivery of a nucleic acid to the nucleus of a cell and thus enhancing expression of the nucleic acid in a subject, comprising administering to the subject a purified complex comprising a nucleic acid bound to a nuclear localization peptide, thereby enhancing delivery of the nucleic acid to the nucleus of the cell of the subject. The herein described known *in vivo* methods of gene delivery can be used in conjunction with this method to deliver the complex to the cells. In particular, liposomes, and preferably cationic liposomes, can be administered with the complex. Furthermore, means known in the art for targeting a specific organ and/or cell type, such as cancer cells, can be utilized.

The present invention additionally provides a method of enhancing delivery of a nucleic acid to mitochondria of a cell, comprising administering to the cell a complex comprising the nucleic acid bound to a mitochondrial localization peptide, thereby enhancing the delivery of the nucleic acid to the mitochondrion of the cell. This method, as also described herein for nuclear localization, can be performed in conjunction with known delivery methods, with cationic liposomes being particularly useful. Administration of the complex can be achieved identically to that for nuclear localization. An example of a mitochondrial localization peptide that can be useful in this method is that set forth in SEQ ID NO:5.

Means of administration can be comparable to that already described for known *in vivo* methods for delivery of nucleic acids. Briefly, the complex (and any additional components, as described herein) can be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, by inhalation, or the like, although intravenous or inhalation administration is typically preferred.

The amount of active compound administered will, of course, be dependent on the subject being treated, the subject's weight, the manner of administration and the judgment of the prescribing physician. Generally, however, dosage will approximate that which is typical for the administration of nucleic acids, particularly those that remain extrachromosomal, and can preferably be in the range of about 270 µg DNA, 9 µg peptide and 900 µg liposome/kg about once every two to three weeks.

Depending on the intended mode of administration, the pharmaceutical compositions may be in the form of solid, semi-solid or liquid dosage forms, such as, for example, tablets, suppositories, pills, capsules, powders, liquids, suspensions, lotions, creams, gels, or the like, preferably in unit dosage form suitable for single administration of a precise dosage. The compositions will include, as noted above, an effective amount of the selected complex in combination with a pharmaceutically acceptable carrier and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc.

For solid compositions, conventional nontoxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talc, cellulose, glucose, sucrose, magnesium carbonate, and the like. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc. an active compound as described herein and optional pharmaceutical adjuvants in an excipient, such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like, for example, sodium acetate, sorbitan monolaurate, triethanolamine sodium acetate, triethanolamine oleate, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington's Pharmaceutical Sciences*, E. W. Martin, (ed.), Mack Publishing Co., Easton, PA.

Parenteral administration, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system, such that a constant level of dosage is maintained. See, e.g., U.S. Patent No. 3,710,795, which is incorporated by reference herein.

The exact amount of such compounds required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease that is being treated, the particular compound used, its mode of administration, and the like. Thus, it is not possible to specify an exact activity promoting amount.

By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected complex without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

The present invention further provides a method of enhancing expression of a transfected nucleic acid in a cell in a plant comprising administering to the plant a complex comprising the nucleic acid bound to a plant nuclear localization peptide, thereby enhancing expression of the nucleic acid in the cell in the plant. Plant nuclear localization sequences are known in the art, have the same characteristics as other nuclear localization sequences, and can be utilized herein as a plant nuclear localization peptide so long as it, by definition herein, directs delivery to the nucleus in plant cells. Therefore, plant nuclear localization peptides can be selected as described herein for any nuclear localization peptide. A minimum plant localization peptide is therefore the peptide listed herein as SEQ ID NO:6. Another example of a useful plant nuclear

localization peptide is listed herein as SEQ ID NO:4, which is derived from *Argobacterium tumefaciens*.

Administration and dosages can be as already utilized for delivery/transfection of nucleic acids to cells of plants. Dosage will depend upon the purpose of the delivered nucleic acid and will typically range from about 3 $\mu g/10^5$ cells to about 40 $\mu g/10^5$ cells.

Statement Concerning Utility

One of the obstacles to the application of gene therapy to any disease is the poor transfection efficiency which makes gene therapy too costly and too labor intensive. Therefore, the present invention, which enhances the delivery of nucleic acids to the nucleus or mitochondria can be very useful for delivery of nucleic acids. Thus, the invention can be useful, for example, for any method or treatment wherein delivery of nucleic acids is desired, as in gene therapy. In particular, one can transport a desirable gene into the nucleus of a target cell, thus, ultimately providing, for example, a protein or peptide encoded by that gene. An example of useful gene therapy for which the present invention would be useful includes delivery and expression of the human cystic fibrosis transmembrane conductance regulator gene for treatment of cystic fibrosis. Additionally, the present invention can be utilized to deliver antisense RNAs, to block the ultimate production of a particular protein.

Furthermore, the present invention provides for delivery of nucleic acids to the mitochondria, which can be useful for, for example, correcting defects in mitochondrial DNA. In addition, the present invention can be used for delivery of nucleic acids *ex vivo* for numerous purposes, such as transfecting desirable genes into cells prior to implantation of the cells in a body, or for producing and harvesting large quantities of an RNA or a protein encoded by a transfected gene from *in vitro* cell cultures. As another example, this invention can be utilized to enhance antisense RNA delivery, for example, to block production of a particular protein or to enhance recovery of a specific RNA in single-stranded isolates from cells by binding to other RNAs.

Many other uses will be apparent to persons of ordinary skill in the art. In plants, the present method can be utilized to deliver an exogenous gene to cells of the plant for production of beneficial proteins in the plant, for example, growth enhancers or proteins to retard spoilage of any edible portions of the plant.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

I. Nuclear Localization

Two nuclear localization (NLS) peptides were synthesized by Research Genetics (Huntsville, Alabama) using standard procedures. Both peptides contained a seven amino acid nuclear localization signal (SV40 T-antigen) and five L-lysine residues. Peptide 1 is listed in SEQ ID NO:2. Peptide 2 (listed in SEQ ID NO:3) contained one additional amino acid (separating the nuclear localization signal from the poly L-lysine tail) which causes the peptide to be less linear.

A. Isolated Nuclei

Isolated, intact cell nuclei from 2CF cells (an epithelial like cell line from a cystic fibrosis patient) and bovine pulmonary artery endothelial cells (BPAECs) isolated directly from arteries (cells passaged about 15 times, then discarded) as described in Ryan, J.W., *et al.*, *Tissue Cell* 10:555-562 (1979), were used to determine if the peptide would enhance nuclear concentrations of fluorescently labeled exogenous DNA. A nuclear extract was prepared by methods standard in the art (Ausubel, F.M., *et al.*, eds., *Current Protocols in Molecular Biology*, Greene Publishing Associates, Inc. and John Wiley and Sons, Inc., p. 12.1.1 - 12.1.6 (1994)). Acridine orange labeled plasmid DNA (1 μg) was combined with 2 μg of NLS peptide (using peptide 1 (listed herein as SEQ ID NO:2) and peptide 2 (listed herein as SEQ ID NO:3) in separate experiments) and incubated for 15 minutes at room temperature. The peptide-DNA

complex was then added to the isolated, intact nuclei and 2 µl of 1 M ATP and 10 µl cellular extract was added, and the mixture was incubated for 30 minutes at 37°C. The nuclei were then collected by centrifugation, washed twice with phosphate-buffered saline, and lysed by the addition of sterile water. The fluorescence of the labeled plasmid was determined at an emission of 526 nm in a fluorometer.

In experiments designed to investigate the effect of increased peptide concentration, several different concentrations of peptide (0.5-4.0 µg) were mixed with the plasmid DNA. Following incubation, the nuclei were collected by centrifugation, and resuspended in phosphate buffered saline. This process was repeated three times and the final nuclear pellet was resuspended and lysed in sterile water containing 0.1% Triton X-100. Nuclear membrane bound components were removed by centrifugation at 12,000 X g and fluorescence was quantified in a fluorometer at an emission of 526 nm. The results from nuclei isolated from 2CF cells are listed in Table 1.

Table 1. Synthetic Peptide-Mediated Uptake of Labeled DNA by Intact Nuclei of 2CF Cells.

Peptide Concentration µg	Acridine Orange-labeled DNA in Nucleus, in Fluorescence at 526 nm	
	Peptide 1	Peptide 2
0	90	90
0.5	134	104
1.0	122	133
2.0	162	157
4.0	156	94

Table 2 shows the peptide 1-mediated uptake of acridine orange-labeled DNA by nuclei isolated from BPAECs. In Experiment 1, DNA alone was incubated with nuclei under the above optimum conditions. Experiment 2 shows uptake when DNA is incubated with 2 µg peptide as described above (i.e., with ATP); Experiment 3

shows uptake with 1 μg DNA plus 2 μg peptide, but without ATP added to the uptake incubation. In Experiment 4, excess non-labeled DNA was added to the uptake incubation. Thus, this method is functional in more than one cell type.

Table 2. Synthetic Peptide 1-Mediated Uptake of Labeled DNA by BPAEC Nuclei.

Experiment No.	Transfection Conditions	Acridine Orange-Labeled DNA in Nucleus, in Fluorescence at 526 nm
1.	DNA	117
2.	DNA + Peptide	125
3.	DNA + Peptide in absence of ATP	104
4.	DNA + Peptide in presence of excess non-labeled DNA	89

B. Cells

NLS peptide assisted liposome gene transfers were performed in BPAECs as follows: Cells were grown to near confluence in a P-100 tissue culture dish. Plasmid DNA (30 μg) (pCMV4-CAT) containing the gene for chloramphenicol acetyltransferase (CAT) driven by a cytomegalovirus promoter was combined with 1 μg of NLS peptide. Following incubation for 15 minutes at room temperature, 0.01, 0.1, 0.25, 0.5, or 1.0 μg of the NLS peptide/plasmid DNA complex was combined with 90 μg of Lipofectin™ (Bethesda Research Laboratories) and added per plate (100 mm) of BPAEC and the cells were incubated overnight at 37°C, in 5% CO_2. Following this incubation the medium was changed and the cells were incubated for an additional 36-40 hours and then harvested for CAT assay.

Cells were harvested by scraping into 3 ml of phosphate buffered 0.1% NaCl and collected by centrifugation. The cell pellet was resuspended in 40 mM Tris-HCl buffer, pH 7.5, containing 10 mM EDTA and 150 mM NaCl and frozen in an

acetone dry ice bath for 5 minutes. The frozen pellet was thawed in a 37°C water bath and the freeze-thaw process was repeated two times. An aliquot (50 µl) of cell suspension was removed and assayed for CAT activity using standard methods (Berger, S. and Kimmel, A.R., eds., *Methods in Enzymology: Guide to Molecular Cloning Techniques*, Vol. 152:717-718 (1987)). The CAT assay was quantified as cpm of radiolabeled acetyl transferred from coenzyme A to chloramphenicol.

Table 3 shows the results of administering to BPAECs 30 µg pCMV4-CAT with varying concentrations of peptide 1. One control as no lipofectin or peptide in the reaction, while another shows the reaction with 90 µg lipofectin and no peptide. The experimental reactions show the results with 1.0, 0.5, 0.25, 0.1, and 0.01 µg peptide 1 to determine successful ratios of DNA to peptide.

Table 3. CAT activity in BPAECs After Liposome Transfer of 30 µg pCMV4-CAT DNA Into Whole Cells With Peptide 1.

Peptide Concentration, µg	Chloroacetylation, cpm	Specific Activity, cpm/µgPr/hr
0 (w/no liposomes)	4.4	1.5
0 (w/ liposomes)	196	46.3
1	676	240
0.5	1104	391.6
0.25	549	194.6
0.1	362	59.4
0.01	211	49.9

A successful ratio was then used in a liposome transfection experiment, the results of which are shown in Table 4. 15 µg of pCMV4-CAT DNA were administered with 1 µg peptide 1. The control experiment has no peptide. The peptide

increases the amount of CAT activity in BPAECs resulting from the transfer of pCMV4-CAT over transfer with no peptide.

Table 4. CAT Activity in BPAECs After Liposome Transfer of 15 µg pCMV4-CAT Into Whole Cells With Peptide 1 at 15:1 DNA: Peptide Ratio.

Transfer Conditions	Chloroacetylation, cpm
Control (no peptide)	68
w/ peptide	108

A nuclear localization peptide comprising the complete histone protein (Boenringer Mannheim) was utilized in a liposome transfer experiment as described above. 1 µg of this nuclear localization peptide was administered with 90 µg lipofectin and with 30 µg of pCMV4-CAT DNA to BPAECs.

Table 5 provides the results from these experiments using complete histone protein.

Table 5. CAT Activity in BPAECs After Liposome Transfer of 1 µg Complete Histone Protein With 30 µg pCMV4-CAT.

Transfer Conditions	% Activity*
Control (DNA only)	not detectable
DNA w/liposomes only	26%
DNA w/liposomes & complete histone protein	29%

*Conversion of unacetylated chloramphenicol to acetylated chloramphenicol.

A nuclear localization peptide comprising polylysine (listed herein as SEQ ID NO:7) was utilized in a liposome transfer experiment as described above. 1 µg of polylysine was complexed with 30 µg of pCMV4-CAT and 90 µg lipofectin and administered to BPAECs.

The results of these polylysine experiments are shown in Table 6 below. These results demonstrate that polylysine alone does not detectably enhance liposome-mediated transfection of nucleic acids.

Table 6. CAT Activity in BPAECs After Liposome Transfer of pCMV4-CAT with Polylysine.

Transfer Conditions	% Activity*
Control (DNA only)	not detectable
DNA w/liposomes only	26%
DNA w/liposomes & polylysine	24%

*Conversion of unacetylated chloramphenicol to acetylated chloramphenicol.

II. Mitochondrial Localization

A mitochondrial localization peptide comprising the amino acid sequence set forth in SEQ ID NO:5 was utilized in a liposome transfer experiment as described above for nuclear localization experiments. 1 μg of this mitochondrial localization peptide was administered with 90 μg lipofectin and 30 μg pCMV4-CAT DNA to BPAECs. CAT activity was then measured, and the results are shown below in Table 7.

Table 7. CAT Activity in BPAECs after Liposome Transfer of pCMV4-CAT with Mitochondrial Localization Peptide.

Transfer Conditions	% Activity*
Control (DNA only)	not detectable
DNA w/liposomes only	26%
DNA w/liposomes & mitochondrial localization peptide	39%

*Conversion of unacetylated chloramphenicol to acetylated chloramphenicol.

Additional experiments were performed on viable cells using a camera with enhanced low light capability and computer control (time lapse) of the shutter on the fluorescent light source. BPAECs were grown on a coverslip which had previously been glued on the underside of a 35 mm tissue culture dish over a 1 cm hole. The cells were cultured for 24 h on cover slips, transfected with acridine orange labeled DNA/liposome complex, with either a nuclear or a mitochondrial localization peptide, at a 1:30 peptide:DNA ratio on a 37°C microscope stage and images of the chosen field were recorded every 2 minutes. During the first 10 minutes of transfection with the nuclear localization peptide (peptide 1 listed herein as SEQ ID NO:2), the DNA fluorescence was limited to abundant, small, well defined regions of the cell periphery. After 24 minutes the fluorescence appeared to be exclusively nuclear and the nucleus of the cell was very well defined against the much darker cytoplasm. If transfection was performed with labeled DNA charge associated with the mitochondrial localization peptide listed herein as SEQ ID NO:5, the nucleus failed to become heavily stained and a region of the cytoplasm appeared to concentrate the stain. Further localization of labeled DNA (complexed with a mitochondrial localization peptide) within the cytoplasm to the mitochondria can be determined by separating subcellular fractions of transfected cells by methods known and standard in the art and measuring relative amounts of label, for example, radioactivity, in each fraction.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Vanderbilt University
(B) STREET: 405 Kirkland Hall
(C) CITY: Nashville
(D) STATE: Tennessee
(E) COUNTRY: United States of America
(F) POSTAL CODE (ZIP): 37240
(G) TELEPHONE: (615) 343-2430
(H) TELEFAX: 615 343-0488
(I) TELEX: None

(ii) TITLE OF INVENTION: COMPOSITIONS FOR AND METHODS OF ENHANCING DELIVERY OF NUCLEIC ACIDS TO CELLS

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Not yet known
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Perryman, David G.
(B) REGISTRATION NUMBER: 33,438
(C) REFERENCE/DOCKET NUMBER: 2200.0191

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 404/688-0770
(B) TELEFAX: 404/688-9880

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..7
(D) OTHER INFORMATION: /note= "Description: nuclear localization peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Pro Lys Lys Lys Arg Lys Val Arg Lys Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Description: peptide 1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Pro Lys Lys Lys Arg Lys Val Lys Lys Lys Lys Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..13

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro Lys Lys Lys Arg Lys Val Leu Lys Lys Lys Lys Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..18
(D) OTHER INFORMATION: /note= "Description: plant nuclear localization peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Arg Pro Arg Glu Asp Asp Asp Gly Glu Pro Ser Glu Arg Lys Arg
1 5 10 15

Glu Arg

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /note= "Description: mitochondrial localization signal"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Leu Phe Asn Leu Arg Ile Leu Leu Asp Asp Ala Ala Phe Arg Asp
1 5 10 15

Gly Lys Lys Lys
20

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..4
(D) OTHER INFORMATION: /label= a
/note= "Description: nuclear localization peptide"

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 2
(D) OTHER INFORMATION: /label= b
/note= "Xaa can equal either Arg or Lys"

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 3
(D) OTHER INFORMATION: /label= c
/note= "Xaa can equal any amino acid"

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 4
(D) OTHER INFORMATION: /label= d
/note= "Xaa can equal either Arg or Lys"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Lys Xaa Xaa Xaa
1

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..6
(D) OTHER INFORMATION: /note= "Description: nuclear localization peptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Lys Lys Lys Lys Lys
1 5

What is claimed is:

1. A purified complex comprising a nucleic acid bound to a nuclear localization peptide.

2. The complex of Claim 1, wherein the nuclear localization peptide has a polylysine tail.

3. The complex of Claim 2, further comprising asialorosomucoid.

4. The complex of Claim 1, further comprising a liposome.

5. The complex of Claim 4, wherein the liposome is a cationic liposome.

6. The complex of Claim 1, wherein the nuclear localization peptide comprises the amino acid sequence listed in SEQ ID NO: 1.

7. The complex of Claim 2, wherein the nuclear localization peptide comprises the amino acid sequence listed in SEQ ID NO:2.

8. The complex of Claim 2, wherein the nuclear localization peptide comprises the amino acid sequence listed in SEQ ID NO:3.

9. The complex of Claim 1, wherein the nuclear localization peptide is the complete histone protein.

10. The complex of Claim 1, wherein the nuclear localization peptide has the amino acid sequence listed in SEQ ID NO:4.

11. The complex of Claim 2, wherein the polylysine tail has about four or five lysine residues.

12. A purified complex comprising a nucleic acid bound to a mitochondrial localization peptide.

13. The complex of Claim 12, wherein the mitochondrial localization peptide has the amino acid sequence listed in SEQ. ID NO:5.

14. The complex of Claim 12, further comprising a liposome.

15. A purified complex comprising a liposome, a nucleic acid and a nuclear localization peptide.

16. A method of enhancing expression of a transfected nucleic acid in a cell comprising administering the complex of Claim 1 to the cell, thereby enhancing expression of the transfected nucleic acid in the cell.

17. A method of enhancing expression of a transfected nucleic acid in a cell comprising administering the complex of Claim 4 to the cell, thereby enhancing expression of the transfected nucleic acid in the cell.

18. A method of enhancing expression of a transfected nucleic acid in a cell comprising administering the complex of Claim 7 to the cell, thereby enhancing expression of the transfected nucleic acid in the cell.

19. A method of enhancing delivery of a nucleic acid to the nucleus of a cell, comprising administering the complex of Claim 1 to the cell, thereby enhancing the delivery of the nucleic acid to the nucleus of the cell.

20. A method of enhancing delivery of a nucleic acid to the nucleus of a cell, comprising administering the complex of Claim 4 to the cell, thereby enhancing the delivery of the nucleic acid to the nucleus of the cell.

21. A method of enhancing delivery of a nucleic acid to the nucleus of a cell, comprising administering the complex of Claim 7 to the cell, thereby enhancing the delivery of the nucleic acid to the nucleus of the cell.

22. A method of enhancing delivery of a nucleic acid to the nucleus of a cell in a subject, comprising administering to the subject the complex of Claim 1, thereby enhancing delivery of the nucleic acid to the nucleus of the cell of the subject.

23. A method of enhancing delivery of a nucleic acid to the nucleus of a cell in a subject, comprising administering to the subject the complex of Claim 4, thereby enhancing delivery of the nucleic acid to the nucleus of the cell of the subject.

24. A method of enhancing delivery of a nucleic acid to the nucleus of a cell in a subject, comprising administering to the subject the complex of Claim 7, thereby enhancing delivery of the nucleic acid to the nucleus of the cell of the subject.

25. A method of enhancing delivery of a nucleic acid to a mitochondrion of a cell, comprising administering to the cell a complex of Claim 12, thereby enhancing the delivery of the nucleic acid to the mitochondrion of the cell.

26. The method of Claim 25, wherein the mitochondrial localization peptide has the amino acid sequence listed in SEQ ID NO:5.

27. A method of enhancing expression of a transfected nucleic acid in a cell in a subject comprising administering to the subject the complex of Claim 1, thereby enhancing expression of the transfected nucleic acid in the cell.

28. The method of Claim 27, wherein the complex is administered by intravenous injection.

29. The method of Claim 27, wherein the complex is administered by inhalation.

30. A method of enhancing expression of a transfected nucleic acid in a cell in a subject comprising administering to the subject the complex of Claim 4, thereby enhancing expression of the transfected nucleic acid in the cell.

31. A method of enhancing expression of a transfected nucleic acid in a cell in a plant comprising administering to the plant a complex comprising the nucleic acid bound to a plant nuclear localization peptide, thereby enhancing expression of the nucleic acid in the cell in the plant.

32. The method of Claim 31, wherein the plant nuclear localization peptide has the amino acid sequence listed in SEQ ID NO:4.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07543

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00; A01N 43/04; A61K 31/70
US CL :435/172.3; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	British Medical Bulletin, Volume 51, Number 1, issued 1995, Schofield et al., "Non-viral approaches to gene therapy", pages 56-71, see entire document.	1-32
Y	Current Opinion in Biotechnology, Volume 5, issued 1994, Ledley, "Non-viral gene therapy", pages 626-636, see entire document.	1-32
A, P	Biotechnology, Volume 13, issued 13 March 1995, Hodgson, "The vector void in gene therapy", pages 222-225, see entire document.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 JULY 1995

Date of mailing of the international search report

15SEP1995

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07543

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	Nucleic Acids Research, Volume 23, Number 1, issued January 1995, Seibel et al., "Transfection of mitochondria: strategy towards a gene therapy of mitochondrial DNA diseases", pages 10-17, see entire document.	12-14, 25-26
Y	Nature Genetics, Volume 5, issued October 1993, Alton et al., "Non-invasive liposome-mediated gene delivery can correct the ion transport defect in cystic fibrosis mutant mice", pages 135-142, see entire document.	4-5, 14-17, 20, 23, 29-30
Y	Accounts of Chemical Research, Volume 26, Number 5, issued 1993, Behr, "Synthetic gene-transfer vectors", pages 274-278, see entire document.	1-32
Y	Trends in Biochemical Sciences, Volume 16, issued December 1991, Dingwall et al., "Nuclear targeting sequences - a consensus?", pages 478-481, see entire document.	1-32
Y	Journal of Biological Chemistry, Volume 262, Number 10, issued 05 April 1987, Wu et al., "Receptor-mediated in vitro gene transformation by a soluble DNA carrier system", pages 4429-4432, see entire document.	2, 3, 11
Y	Journal of Biological Chemistry, Volume 266, Number 22, issued 05 August 1991, Wu et al., "Receptor-mediated gene delivery in vivo", pages 14338-14342, see entire document.	2-3, 11, 22-24, 27-28, 30
Y	Molecular and General Genetics, Volume 239, issued 1993, Rossi et al., "The VirD2 protein of Agrobacterium tumefaciens carries nuclear localization signals important for transfer of T-DNA to plants", pages 345-353, see entire document.	31-32
Y	Journal of Biological Chemistry, Volume 264, Number 21, issued 25 July 1989, Kaneda et al., "Introduction and expression of the human insulin gene in adult rat liver", pages 12126-12129, see entire document.	1-5, 14-17,19, 20, 22, 23, 27-30
X --- Y	WO, A, 93/19768 (SZOKA ET AL.) 14 October 1993, see entire patent application.	1-5, 11, 15-17, 19-20, 22-23, 27-30 --- 6-9, 18, 21, 24

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/07543

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, WPIDS
Search terms: gene or genetic or DNA (W) transfer, delivery or therapy; nuclear localizing or localization; nuclear or nucleus (W) targeting or directed or import?; liposome, proteoliposome, receptor mediated, non-viral vector, fusogenic or karyogenic or karyophilic peptide, nuclear protein or peptide.